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Overexpression of catalase or Bcl-2 delays or prevents alterations in phospholipid metabolism during glucocorticoid-induced apoptosis in WEHI7.2 cells ☆

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Abstract

Dexamethasone-treated WEHI7.2 mouse thymoma cells readily undergo apoptosis. WEHI7.2 variants that overexpress catalase (CAT38) or Bcl-2 (Hb12) show a delay or lack of apoptosis, respectively, when treated with dexamethasone. This is accompanied by a delay or lack of cytochrome *c* release from the mitochondria suggesting that alterations in the signaling phase of apoptosis are responsible for the observed resistance. Because membranes are a rich source of signaling molecules, we have used ^{31}P NMR spectroscopy to compare phospholipids and their metabolites in WEHI7.2, CAT38 and Hb12 cells after dexamethasone treatment. Increased lysophosphatidylcholine (lysoPtdC) content accompanied phosphatidylserine (PtdS) externalization in the WEHI7.2 cells. Both changes were delayed in CAT38 cells suggesting phosphatidylcholine (PtdC) metabolites may play a role in steroid-induced apoptotic signaling. The steroid-resistant Hb12 cells showed a dramatic increase in glycerophosphocholine (GPC) content, suggesting increased phospholipid turnover may contribute to the anti-apoptotic mechanism of Bcl-2.

Keywords: Glucocorticoid; NMR spectroscopy; Apoptosis; Thymoma; Bcl-2; Catalase

1. Introduction

Glucocorticoid-induced apoptosis in lymphocytes is a well-documented process that is important physiologically for the sorting of immature lymphocytes and has been exploited clinically for the treatment of leukemia and lymphoma [1]. Resistance either initially or developed during the course of treatment can limit the use of glucocorticoids as chemotherapeutic agents and, in acute lymphoblastic leukemia, is correlated with poorer prognosis [2]. A decrease in or lack of functional glucocorticoid receptors has been shown to cause resistance in cell culture and in the clinic [3–5]; however, only a portion of the resistance can be attributed to receptor alterations [6–9]. Elucidating the events that occur during the signaling phase of glucocorticoid-induced apoptosis, i.e. after the translocation of the steroid-receptor complex to the nucleus and prior to the release of cytochrome *c* into the cytosol, is key to understanding steroid resistance and developing chemotherapeutic agents that circumvent this resistance.

Abbreviations: AA, arachidonic acid; AAPtdC, 1-alkyl-2-acyl-phosphatidylcholine; AAPtdE, 1-alkyl-2-acyl-phosphatidylethanolamine; CAT38, catalase-overexpressing WEHI7.2 cells; CDP-choline, cytidine diphosphate-choline; *ck*, choline kinase; CL, cardiolipin; CoA, coenzyme A; COX-2, cyclooxygenase 2; *CPT*, phosphocholine diacylglycerol transferase; *ct*, phosphocholine cytidylyltransferase; DAG, diacylglycerol; DEX, dexamethasone; ETOH, ethanol; FA, fatty acid; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPG, glycerophosphoglycerol; GPGPG, bis(glycerol 3-phospho)glycerol; Gro-3-P, glycerol 3-phosphate; Hb12, Bcl-2-overexpressing WEHI7.2 cells; lysoPtdC, lysophosphatidylcholine; NSAID, non-steroidal anti-inflammatory drug; NTP, nucleoside triphosphate; PA, phosphatidate; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PC, phosphocholine; PC-L, phosphocholine-containing lipids; PDE, phosphodiester; PE, phosphoethanolamine; PE-L, phosphoethanolamine-containing lipids; P_i, inorganic phosphate; *PLA*$_2$, phospholipase A$_2$; *PLC*, phospholipase C; *PLD*, phospholipase D; PME, phosphomonoester; PtdC, phosphatidylcholine; PtdE, phosphatidylethanolamine; PtdE$_{plasm}$, phosphatidylethanolamine plasmalogen; PtdG, phosphatidylglycerol; PtdI, phosphatidylinositol; PtdS, phosphatidylserine; ROS, reactive oxygen species; SM, sphingomyelin

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WEHI7.2 mouse thymoma cells treated with dexamethasone, a synthetic glucocorticoid, readily undergo apoptosis. Enhancing the endogenous antioxidant defense in WEHI7.2 cells through overexpression of catalase or thioredoxin or selection for resistance to hydrogen peroxide results in WEHI7.2 variants that are protected from steroid-induced apoptosis [10–12]. The degree of protection is correlated with a delay or lack of cytochrome *c* release into the cytosol. Overexpression of Bcl-2, a protein which protects against apoptosis by reactive oxygen species (ROS) [13–16] and has been shown to alter the redox status in some cell types [17], also protects against glucocorticoid-induced apoptosis in WEHI7.2 cells [18]. Taken together, these data suggest that signaling pathways sensitive to ROS or the products of ROS-induced damage may be critical to steroid-induced apoptosis.

Membranes are a rich source of signaling molecules and a major target of ROS. There is some evidence that lipid peroxidation occurs after steroid treatment of lymphocytes [13,19]. Further, treatment of WEHI7.2 cells with a vitamin E analog protects the cells from steroid-induced apoptosis [20]. A direct attack by ROS on membranes can result in the release of lipid peroxidation products that damage proteins and DNA and alter membrane phospholipid composition [21]. Perturbations in membrane phospholipid composition may be important for both phospholipid and membrane-bound protein signaling molecules [22]. Dexamethasone treatment alters membrane fluidity in some systems [23,24] and has been shown to suppress phospholipid synthesis [25,26] suggesting that steroid treatment may affect membrane phospholipid composition. Alternatively, oxidative stress has been shown to stimulate production of ceramide [27], a sphingolipid metabolite that is a well-documented apoptotic signal [28]. Steroids have been reported to stimulate ceramide generation in thymocytes as part of the apoptotic cascade [29].

The goal of this study was to identify alterations in phospholipids or phospholipid metabolites due to dexamethasone treatment that differ depending on the steroid sensitivity of the WEHI7.2 variants. To generate a global picture of phospholipid perturbations, we have used ^{31}P NMR spectroscopy because this method allows us to examine a large number of phospholipids and their metabolites in one sample. We have focused on steroid-induced phospholipid changes in three WEHI7.2 variants with different steroid sensitivities: (1) WEHI7.2 cells, the parental steroid-sensitive cells; (2) CAT38 cells, WEHI7.2 cells expressing 1.4-fold parental cell catalase activity, that show delayed apoptosis after dexamethasone treatment [11]; and (3) Hb12 cells, WEHI7.2 cells overexpressing Bcl-2 [18], that show no markers of apoptosis after a 48-h steroid treatment. Measurement of alterations over time after dexamethasone treatment has also allowed us to identify pathways potentially involved in signaling during steroid-induced apoptosis in these cells. Portions of this work have been published in abstract form [30].

2. Materials and methods

2.1. Cell culture and drug treatment

WEHI7.2 mouse thymoma cells [31] were obtained from Dr. Roger Miesfeld (University of Arizona, Tucson, AZ). Cells were cultured in Dulbecco's Modified Eagle Medium-low glucose (GIBCO-BRL Products, Grand Island, NY) supplemented with 10% calf serum (Hyclone Laboratories, Logan, UT) at 37 °C in a 5% CO_2 humidified environment. These cells were maintained in exponential growth at a density between 0.02 and 2×10^6 cells/ml. WEHI7.2 cells stably transfected with and overexpressing human Bcl-2 (Hb12), also obtained from Dr. Miesfeld, were constructed and maintained as described by Lam et al. [32]. Catalase-overexpressing WEHI7.2 cells (CAT38) were constructed and cultured in the presence of 800 μg/ml G418 (GIBCO-BRL) to ensure continued catalase expression as described in Tome et al. [11]. CAT38 cells were subcultured in the absence of selection drug for 1 week prior to sampling. Immediately before each experiment, live cells were separated from those that underwent spontaneous apoptosis using Ficoll-Plaque Plus reagents and the manufacturer's suggested protocol (Amersham Pharmacia Biotech, Piscataway, NJ). Cells were subcultured into medium and incubated in the presence of 1 μM dexamethasone (Sigma Chemical Co., St. Louis, MO) or ethanol as a vehicle control (final conc. = 0.01%) for indicated times before harvest.

2.2. Annexin binding and cell cycle analysis

Cells were harvested by centrifugation ($500 \times g$ for 5 min) and washed twice with PBS. For % apoptosis, the cells were incubated with annexin V and propidium iodide according to the manufacturer's instructions (Apoptosis Detection Kit, R&D Systems, Minneapolis, MN). Cells that were annexin V-positive and propidium iodide-negative were scored as apoptotic. For cell cycle analysis, the cells were resuspended in Krishan's Buffer (0.1% sodium citrate; 0.02 mg/ml RNase; 0.3% IGEPAL; 50 μg/ml propidium iodide) and incubated overnight at 4 °C. Both types of samples were analyzed using a FACScan flow cytometer with CellQuest software (Becton Dickinson, San Jose, CA). Ten thousand cells were analyzed per sample. Samples from exponentially growing untreated cell cultures 24 h after subculture were used as control samples.

2.3. ^{31}P NMR spectroscopy

Approximately, $6–8 \times 10^8$ cells from cultures at a density of $7–8 \times 10^5$ cells/ml were harvested for each time point. NMR samples were prepared, NMR data acquired and data processed as described by Lutz et al. [33]. Briefly, cells were harvested by centrifugation, washed and fixed by adding methanol. Chloroform and water were added to

the sample, then the phases separated by incubation at −20 °C overnight and subsequent centrifugation. Solvents were evaporated from the aqueous and organic phase samples and the aqueous phase lyophilized. Samples were stored at −80 °C until analysis. The lipid sample was dissolved in a ^{2}H-chloroform–methanol–water solution containing 1,2-diamine-tetraacetic acid and the lyophilizate was dissolved in a D_2O solution containing 50 mM 1,2-diamine-tetraacetic acid. ^{31}P NMR spectra were obtained at 202.5 MHz on a vertical, narrow-bore 11.7T Bruker Avance DRX 500 FT NMR spectrometer (Bruker, Rheinstetten, Germany) using a broadband probe for 5 mm tubes. Signals were assigned as described previously [33], except for glycerophosphoglycerol (GPG). GPG and bis(glycerol 3-phospho)glycerol (GPGPG), which have similar chemical shifts, were obtained by saponification of phosphatidylglycerol and cardiolipin, respectively [34]. Spiking the sample with GPG or GPGPG resulted in the exact superimposition of the GPG signal on the unidentified phosphodiester (PDE) region peak. Total sample protein was measured by using the BCA Protein Assay Kit (Pierce Chemical Company, Rockford, IL) as described in Ref. [33]. Metabolite concentrations were expressed as nmol/mg cellular protein.

2.4. Statistics

Fisher's protected least significant difference test for multiple comparisons, a type of ANOVA, was used to test for significant differences between samples (StatView, SAS Institute, Cary NC). Samples were considered significantly different if $P \leq 0.05$. Values reported are mean ± S.E. ($n = 3$ or 4). The difference in % apoptosis was calculated by (% apoptosis + DEX) − (% apoptosis − DEX); then this difference was correlated to [(phospholipid + DEX) − (phosphoospholipid − DEX)]. Regression analyses, using the algorithm in Excel (Microsoft Corporation, Redmond, WA), were used to calculate correlation coefficients (r).

3. Results

3.1. Phosphatidylserine (PtdS) exposure (% apoptosis) and cell cycle analysis

Exposure of PtdS on the external surface of the plasma membrane has been used as a marker of apoptosis [35]. Fig. 1A shows a comparison of PtdS exposure in WEHI7.2, CAT38 and Hb12 cells as a measure of apoptosis after dexamethasone treatment. This information provides a reference point for the other phospholipid changes described in this study. WEHI7.2 cells showed significantly increased apoptosis by 16-h post-dexamethasone treatment. The CAT38 cells showed a delayed increase in PtdS exposure (28 h after dexamethasone treatment), while Hb12 cells did not show an increase in PtdS exposure over this time course. In WEHI7.2 cells, we see a small amount of cytochrome c release into the cytosol by 16-h post-treatment and substantial release by 24 h [11]. CAT38 cells do not show increased cytosolic cytochrome c release until 32 h after treatment [11]. Release of cytochrome c occurs prior to an increase in the number of cells displaying apoptotic morphology in each variant [11]. Significant increases in PtdS occurred slightly before or concomitant with cytochrome c release into the cytosol. This indicates that increased PtdS exposure preceded or coincided with

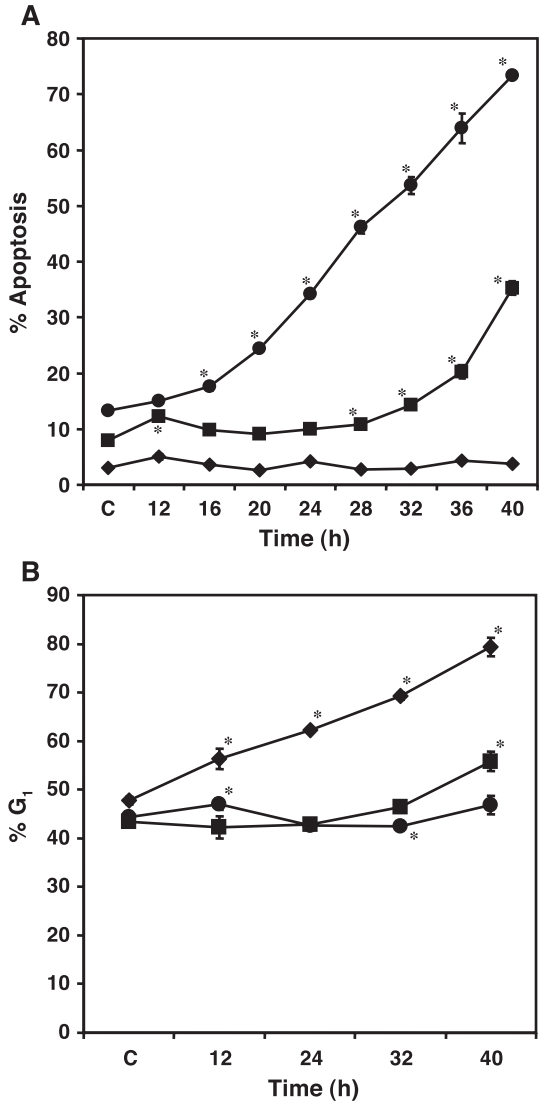


Fig. 1. Apoptosis and G_1 accumulation in the WEHI7.2 (●) cell culture compared to CAT38 (■) and Hb12 (◆) cell cultures after the addition of dexamethasone (time = 0). (A) Each symbol represents the mean percentage of the cells that are annexin V positive and propidium iodide negative ± S.E. ($n = 3$). Where error bars are not visible, they are contained within the symbol. This is a representative experiment which has been replicated. (*) denotes significantly greater than control values (C) for the same cell variant ($P \leq 0.05$). (B) Each symbol represents the mean percentage of cells in G_1 ± S.E. ($n = 3$). (*) Denotes significantly greater than cells cultured in the absence of dexamethasone for the same time ($P \leq 0.05$).

cellular commitment to apoptosis. Spontaneous apoptosis after 24 h in untreated cell cultures (C values) was also significantly different. The WEHI7.2 cells showed the greatest percentage of apoptosis, CAT38 an intermediate amount and Hb12 cells the least.

Glucocorticoid treatment also causes a slowing of the cell cycle in lymphocytes resulting in the accumulation of cells in G_1 [36]. Fig. 1B shows the effect of dexamethasone treatment on G_1-phase accumulation. *C* values are the relative number of cells in G_1 after 24 h in culture in the absence of dexamethasone for comparison. In the presence of dexamethasone, WEHI7.2 cells showed little change in the percentage of cells in G_1. CAT38 cells accumulated in G_1 after 40 h in dexamethasone while a significant increase in the percentage of cells in G_1 was already apparent by 12 h after the addition of dexamethasone to Hb12 cell cultures.

3.2. Comparison of phospholipid alterations to apoptosis and G_1

We have used ^{31}P NMR spectroscopy to quantify multiple phospholipids and their metabolites at several time points during culture in the presence and absence of dexamethasone. Fig. 2 shows typical ^{31}P NMR spectra for both aqueous (A) and organic (B) phases of the sample to illustrate the metabolites that were quantifiable in these samples.

As an indication of the importance of each metabolite in apoptosis, we correlated the change in % apoptosis with

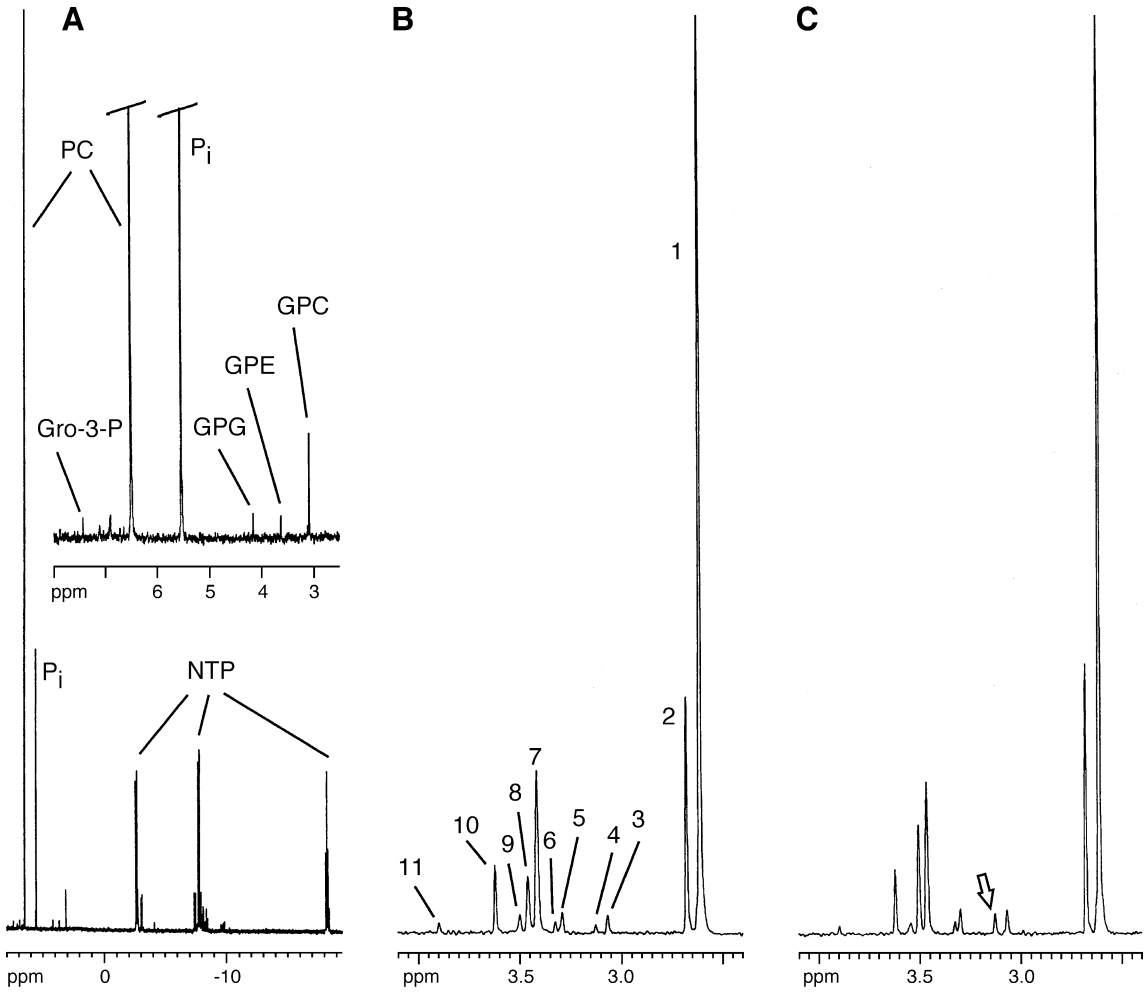


Fig. 2. Characteristic ^{31}P NMR spectra of extracts prepared from WEHI7.2 cells. (A) Aqueous-phase spectrum of a 24-h ETOH-treated sample. The inset represents the expanded low-field region of the spectrum to illustrate some of the metabolites seen at a lower concentration. Abbreviations are as follows: PC, phosphocholine; P_i, inorganic phosphate; NTP, nucleoside triphosphate; Gro-3-P, glycerol 3-phosphate; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPG, glycerophosphoglycerol. (B) Phospholipid spectrum from a 24-h ETOH-treated sample. Peaks are assigned as follows: (1) phosphatidylcholine; (2) 1-alkyl-2-acyl-phosphatidylcholine; (3) phosphatidylinositol; (4) lysophosphatidylcholine; (5) phosphatidylserine; (6) sphingomyelin; (7) phosphatidylethanolamine; (8) phosphatidylethanolamine plasmalogen; (9) 1-alkyl-2-acyl-phosphatidylethanolamine; (10) cardiolipin; (11) phosphatidylglycerol. (C) Phospholipid spectrum from a 24-h dexamethasone-treated sample. The arrow indicates the alteration in peak height of lysophosphatidylcholine in these samples.

Table 1
Correlation of phospholipids and phospholipid metabolites to % apoptosis and % G_1

	Lipid	CORR (r)		Lipid	CORR (r)
% Apoptosis[a] vs.	LysoPtdC	0.9189*	% G_1 vs.	PDE	0.8913*
	SM	0.8713*		GPC	0.8877*
	PC	−0.7583*		GPE	0.8782*
	AAPtdEpl	0.7529*		PME/ΣPDE	−0.6114*
	Ptot	0.7468*		AAPtdE	0.5674*
	AAPtdC	0.7099*		PC	0.5014*
	PtdC	0.7057*		AAPtdEpl	0.5007*
	PME/ΣPDE	−0.6702*		GPG	0.4864*
	PdtI	0.6671*		Ptot	0.1581
	PtdS	0.5900		PtdS	−0.1190
	GPG	−0.4888		SM	−0.1028
	GPE	−0.2396		PdtI	−0.0833
	PDE	−0.2258		AAPtdC	0.0492
	AAPtdE	−0.2107		PtdC	0.0313
	GPC	−0.1701		LysoPtdC	−0.0331

[a] The percentage of apoptosis in the absence of drug was subtracted from the percentage of apoptosis in the presence of drug. This difference was then correlated to the difference in each phospholipid or metabolite (phospholipid in the presence of drug minus phospholipid in the absence of drug).

* Denotes significant correlation ($P \leq 0.05$).

the change in the amount of each metabolite as shown in Table 1. We also compared the percentage of cells in G_1 with individual metabolite concentrations. Lysophosphatidylcholine (lysoPtdC) showed the greatest positive correlation with % apoptosis and the phosphodiesterase (PDE) showed the greatest positive correlation with the percentage of cells in G_1 when all the cell variants and time points were examined together.

However, an overall analysis can obscure important cell specific differences. For example, glycerophosphocholine (GPC) was highly correlated with % G_1 in Hb12 cells ($r = 0.9238$), but in WEHI7.2 cells it was highly correlated with % apoptosis ($r = 0.8665$) and not at all with % G_1 ($r = -0.5338$). The WEHI7.2 cells primarily underwent apoptosis in response to dexamethasone treatment and Hb12 cells primarily accumulated in G_1. This makes it possible to separate phospholipid changes into those that are correlated with apoptosis, those that are correlated with accumulation in G_1 and those that depend on treatment by examining the variants independently. To determine which lipid changes correlated with the onset of apoptosis in the sensitive cells and identify alterations that occur shortly after the addition of dexamethasone (potentially involved in apoptosis signaling), it was necessary to examine some of the metabolites in more detail.

3.3. Choline phospholipids

Although phosphatidylcholine (PtdC), the most abundant membrane lipid, is maintained at a relatively constant percentage in cells, the dynamic flux of PtdC cycling (Fig. 3A) is critical for producing other lipids and maintaining cell viability [37–39]. Perturbations in PtdC synthesis, degradation and remodeling can cause apoptosis in many cell types (e.g. Refs. [40–46] and others).

Both PtdC and lysoPtdC were altered by dexamethasone treatment in the steroid-sensitive cells (Figs. 2C and 3B). LysoPtdC content was significantly increased 12 h after dexamethasone addition in the steroid-sensitive WEHI7.2 cells and continued to increase out to 32-h post treatment. In CAT38 cells the increase was delayed, not appearing until 32 h in dexamethasone and continuing to increase out to 40 h, corresponding to the delay in the appearance of apoptotic markers in these cells. In the steroid-resistant Hb12 cells no consistent change was observed. In the absence of dexamethasone, CAT38 cells showed an increase in lysoPtdC after 40 h in culture which corresponded to an increase in spontaneous apoptosis ($8.47 \pm 0.12\%$ in control vs. $18.03 \pm 0.69\%$ at 40 h), under these culture conditions. PtdC (Fig. 3B) also increased significantly after dexamethasone treatment in the WEHI7.2 cells and slightly at 40 h after dexamethasone treatment in CAT38 cells when the cells were undergoing increased apoptosis.

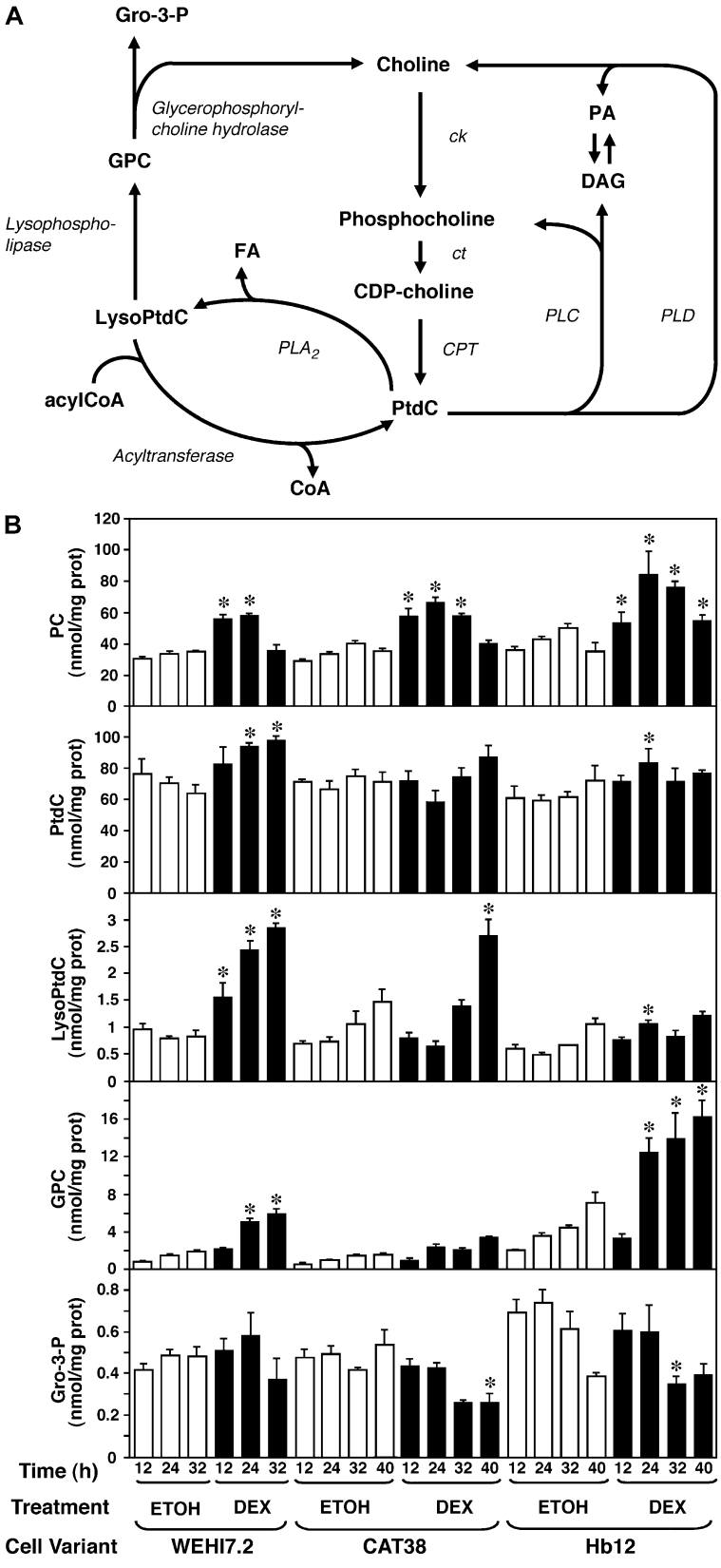
WEHI7.2 cells showed increased GPC, a PtdC degradation product, in the presence of dexamethasone. CAT38 cells showed an increased GPC in the presence of dexamethasone as well, but to a lesser extent. In contrast, Hb12 cells showed a dramatic increase in GPC in the presence of dexamethasone and a more modest increase in GPC in the absence of dexamethasone. The GPC concentration in the Hb12 cells seen after 24 h in the absence of drug was still greater that the GPC concentration in the WEHI7.2 cells after 32 h in the presence of dexamethasone.

Dexamethasone treatment also affected glycerol 3-phosphate (Gro-3-P) and phosphocholine (PC) independent of the steroid sensitivity of the cells. Gro-3-P can be a product of phospholipid degradation, but is also a precursor of diacylglycerol (DAG) or cardiolipin (CL) biosynthesis and a glycolytic intermediate. All three cell variants generally showed a decrease in Gro-3-P in response to dexamethasone treatment. Phosphocholine (PC), a product of choline kinase during PtdC synthesis, can also be a by-product of PtdC hydrolysis by PC-dependent phospholipase C (PLC) or sphingomyelin (SM) breakdown. Initially, dexamethasone caused an increase in intracellular PC in all three cell variants, but by the later time points the levels were similar to those in control (ETOH) cells. No cytidine diphosphate-choline (CDP-choline) was detected in these spectra and quantifiable choline was not seen in the ^{1}H spectra. The correlation of choline lipid alterations to increased PtdS exposure suggests that choline lipid metabolites may be important to the mechanism of steroid-induced lymphocyte apoptosis.

3.4. Phosphomonoesters (PME) and PDE

The relative amount of PME vs. PDE components in cells is used as an indicator of the biological status of cells (see Ref. [37] for review). For example, a relative increase

in PC, one PME component, is correlated with increased proliferation in a number of studies [37]. Relative increases in PDE have been linked to multiple cellular events including cell cycle arrest, chemotherapeutic response [37,46], changes in osmolyte demand [47] and overexpression of Nm-23 [48]. We have compared individual PME



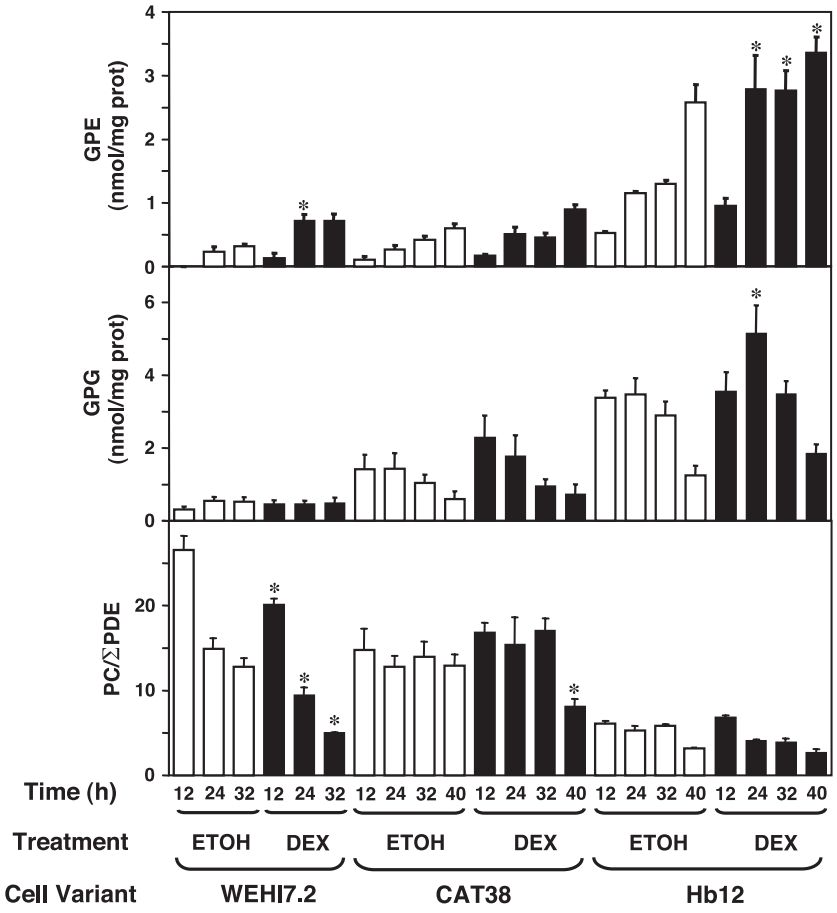


Fig. 4. Comparison of phosphodiester concentrations in WEHI7.2, CAT38 and Hb12 cells over time in the presence and absence of dexamethasone. Abbreviations are: PDE, phosphodiester; and as in Fig. 2. Each bar represents the mean + S.E. ($n = 3$ or 4). (*) Denotes significantly different from the ETOH-treated sample of the same cell variant at the same timepoint ($P \leq 0.05$).

and PDE components as well as a PME/PDE ratio to assess phospholipid metabolism in response to dexamethasone treatment.

The two major PME usually seen in mammalian cells are PC and phosphoethanolamine (PE). In this study, the only quantifiable phospholipid PME was PC, which has been discussed above. Phosphatidylethanolamine (PtdE) can be synthesized and degraded similar to PtdC [37] and was present at ~ 20 nmol/mg prot in the three cell variants in the presence and absence of dexamethasone, except that the WEHI7.2 32-h ETOH sample contained significantly less (16.21 ± 1.4 nmol/mg prot) than the 32-h dexamethasone-treated sample (21.43 ± 0.72 nmol/mg prot). PE, the PtdE metabolite analogous to PC, was not detectable in these samples.

Three PDE peaks were quantifiable in the samples. In addition to GPC, the PtdC degradation product discussed above, glycerophosphoethanolamine (GPE) and GPG, were seen in these samples (Fig. 4). GPE is the breakdown product of PtdE, analogous to GPC in PtdC metabolism. GPE increased in all three cell variants in response to dexamethasone treatment. As with GPC, the greatest amount of GPE was seen in the Hb12 cells, particularly in the presence of dexamethasone. GPE concentration also increased over time in the control (ETOH) samples in all three variants, but not to as great an extent. GPG, a phosphatidylglycerol (PtdG) and CL breakdown product, was also higher in CAT38 and Hb12 cells, in particular, compared to the WEHI7.2 cells.

The ratio of PC to ΣPDE in the three variants is shown in Fig. 4 (bottom panel). PC/ΣPDE decreased over time in the

Fig. 3. Comparison of phosphatidylcholine and related metabolites in WEHI7.2, CAT38 and Hb12 cells over time in the presence and absence of dexamethasone. (A) Choline lipid metabolism in mammalian cells [37,51]. Abbreviations are as follows: CDP-choline, cytidine diphosphate-choline; DAG, diacylglycerol; PA, phosphatidate; FA, fatty acid; CoA, coenzyme A; PtdC, phosphatidylcholine; LysoPtdC, lysophosphatidylcholine; GPC, glycerophosphocholine; Gro-3-P, glycerol 3-phosphate; *ck*, choline kinase; *ct*, phosphocholine cytidylyltransferase; *CPT*, phosphocholine diacylglycerol transferase; PLA_2, phospholipase A_2; *PLC*, phospholipase C; *PLD*, phospholipase D. (B) Choline lipid metabolites and Gro-3-P over time in the presence or absence of dexamethasone. Abbreviations are as in Figs. 2 and 3. Each bar represents the mean metabolite concentration + S.E. ($n = 3$ or 4). (*) Denotes significantly different from the ETOH-treated sample of the same cell variant at the same time point ($P \leq 0.05$).

WEHI7.2 cells in the presence and absence of dexamethasone. However, after 12 h in dexamethasone the value was significantly lower than in the absence of drug (12-h ETOH) and continued to drop out to 32 h as the cells were undergoing apoptosis. CAT38 cells in the control and early dexamethasone-treated time points had a ratio similar to WEHI7.2 cells after 24 h in the absence of drug. The ratio dropped in the 40-h dexamethasone-treated CAT38 sample as the cells were undergoing apoptosis and accumulating in G_1. Hb12 cells had a very low PC/ΣPDE ratio compared to the other two cell variants primarily due to the increased PDE content of these cells. These data suggest that: (1) PC/ΣPDE drops as the cells are undergoing apoptosis and/or accumulating in G_1, and (2) increased resistance to steroid-induced apoptosis is correlated with an increased concentration of PDE.

3.5. Ether-linked phospholipids

Alterations in ether-linked lipid concentrations can alter susceptibility to ROS and blockage of ether-linked lipid remodeling can induce apoptosis [40,49,50]. To determine whether dexamethasone affected the ether-linked lipids, we examined the alterations in the three ether-linked phospholipids quantifiable in these spectra: 1-alkyl-2-acyl-phosphatidylcholine (AAPtdC); 1-alkyl-2-acyl-phosphatidylethanolamine (AAPtdE); and phosphatidylethanolamine plasmalogen ($PtdE_{plasm}$). As shown in Fig. 5, AAPtdC was significantly increased in the WEHI7.2 cells after 24 h in the presence of dexamethasone and was further increased by 32-h post treatment. Neither CAT38 nor Hb12 cells showed any consistent changes in this phospholipid. $PtdE_{plasm}$, a major phospholipid in mitochondrial membranes [51], increased in the presence of dexamethasone in all three cell variants, although the increase was seen earlier in the steroid-sensitive WEHI7.2 cells. AAPtdE, the precursor to $PtdE_{plasm}$, increased in all three cell variants over time independent of drug treatment. The greatest increase was seen in the Hb12 cells.

3.6. PtdS, phosphatidylinositol (PtdI), SM

Although PtdS, PtdI and sphingomyelin (SM) are minor membrane components, these phospholipids may be involved in signaling during apoptosis [29,35,52]. PtdS

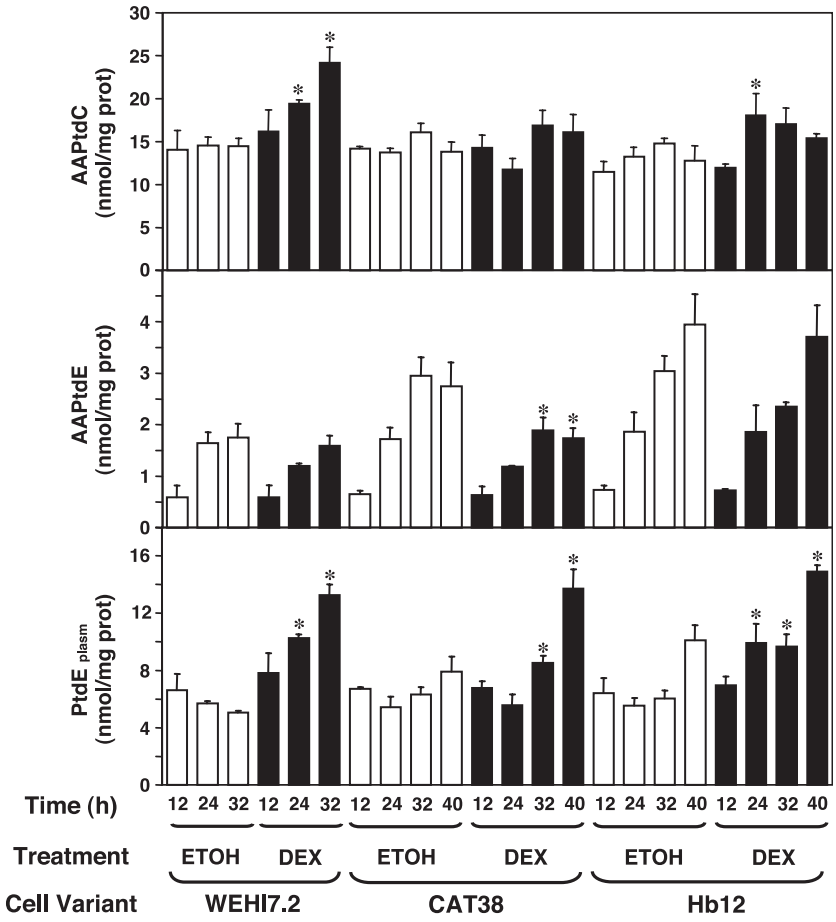


Fig. 5. Comparison of ether-linked lipid concentrations in WEHI7.2, CAT38 and Hb12 cells over time in the presence and absence of dexamethasone. Abbreviations are: AAPtdC, 1-alkyl-2-acyl-phosphatidylcholine; AAPtdE, 1-alkyl-2-acyl-phosphatidylethanolamine; $PtdE_{plasm}$, phosphatidylethanolamine plasmalogen. Each bar represents the mean metabolite concentration + S.E. ($n = 3$ or 4). (*) Denotes significantly different from the ETOH-treated sample of the same cell variant at the same timepoint ($P \leq 0.05$).

significantly increased in WEHI7.2 cells after dexamethasone treatment (Fig. 6). There was a slight increase in PtdS by 40 h in the CAT38 cells, but not of the magnitude seen in the WEHI7.2 cells. PtdI also significantly increased in WEHI7.2 cells by 12 h in dexamethasone and was significantly greater in CAT38 cells by 40 h after drug treatment. Sphingomyelin significantly increased in WEHI7.2 cells by 32 h and in CAT38 cells by 40 h after dexamethasone addition.

3.7. Mitochondrial lipids

Mitochondria are critical in steroid-induced lymphocyte apoptosis because the release of cytochrome *c* into the cytosol is considered the committed step in this pathway [53–55]. Cardiolipin content [56] and oxidation of CL may play a role in cytochrome *c* release [57]. CL concentration was significantly increased in WEHI7.2 cells 24 h after dexamethasone treatment (24-h ETOH 2.66 ± 0.11 vs. 24-h DEX 3.686 ± 0.13 nmol/mg prot) and remained elevated after 32 h in the presence of drug. CL in CAT38 and Hb12 cells showed no significant dexamethasone effect. Phosphatidylglycerol, the CL precursor, did not show significant alterations due to dexamethasone treatment. However, CAT38 and Hb12 cells did show a significant decrease in PtdG from 12 to 40 h in culture independent of drug treatment (data not shown).

3.8. General phospholipid parameters

In WEHI7.2 cells there was a significant increase in total phospholipid/mg prot after dexamethasone treatment that was not seen in the absence of steroids (Fig. 7). CAT38 cells, in the presence of dexamethasone, showed a tendency toward higher total phospholipids by 40 h. There were also significant differences in the PC-containing lipids (PC-L) vs. the PE-containing lipids (PE-L). WEHI7.2 cells had a significantly higher PC-L/PE-L ratio at 12-h ETOH than CAT38 cells and CAT38 cells were significantly higher than Hb12 cells. This relationship was maintained for each time point. This ratio decreased over time in each cell variant in the presence or absence of dexamethasone. The entire data set including each parameter expressed as nmol/mg prot and

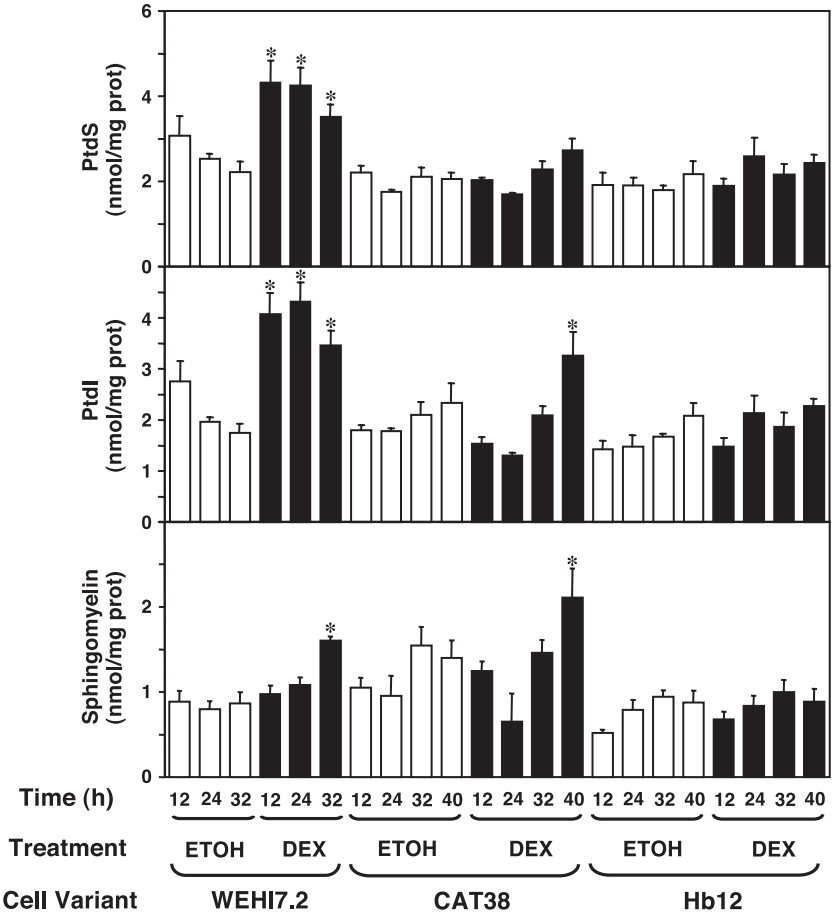


Fig. 6. Comparison of phosphatidylserine, phosphatidylinositol and sphingomyelin concentrations in WEHI7.2, CAT38 and Hb12 cells over time in the presence and absence of dexamethasone. Abbreviations are: PtdS, phosphatidylserine and PtdI, phosphatidylinositol. Each bar represents the mean metabolite concentration + S.E. ($n = 3$ or 4). (*) Denotes significantly different from the ETOH-treated sample of the same cell variant at the same timepoint ($P \leq 0.05$).

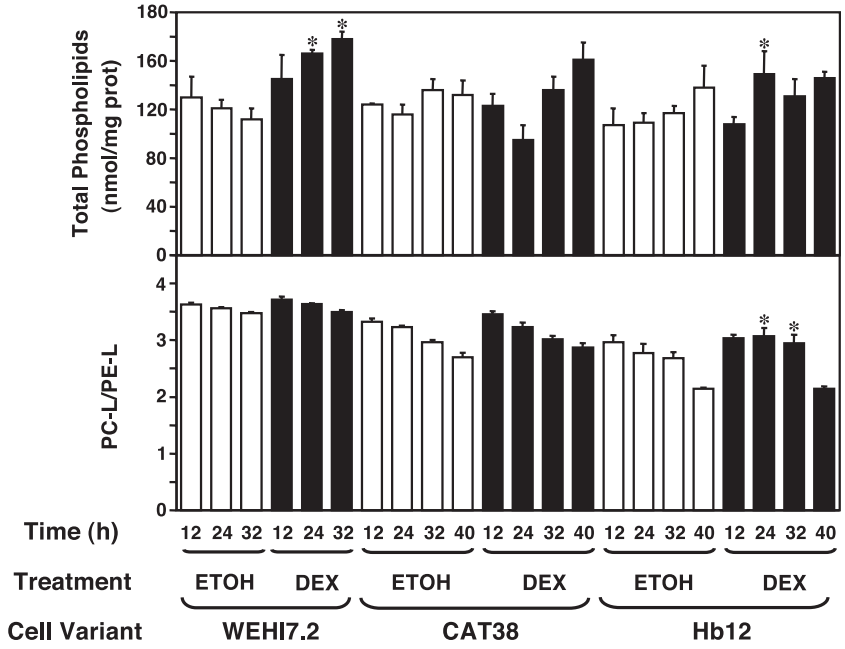


Fig. 7. Comparison of the total phospholipid concentration and the relative amounts of choline-containing to ethanolamine-containing lipids in WEHI7.2, CAT38 and Hb12 cells over time in the presence and absence of dexamethasone. Abbreviations are: PC-L, phosphocholine-containing lipids; PE-L, phosphoethanolamine-containing lipids. Each bar represents the mean + S.E. ($n = 3$ or 4). (*) Denotes significantly different from the ETOH-treated sample of the same cell variant at the same time point ($P \leq 0.05$).

% total sample phosphate is available at http://azcc.biosci.arizona.edu/NMRdata/Index.html.

4. Discussion

4.1. LysoPtdC and apoptosis

Dexamethasone treatment causes multiple alterations in phospholipid metabolism, several of which precede the appearance of other markers of apoptosis. This suggests that metabolites from these pathways may be critical during the signaling phase of steroid-induced lymphocyte apoptosis. In particular, the increases seen in lysoPtdC after steroid treatment indicate that PtdC metabolism may play a role in this process. LysoPtdC increases are correlated with the steroid sensitivity of the WEHI7.2 variants such that the increase is seen earliest in the steroid-sensitive WEHI7.2 cells, is delayed in the CAT38 cells and shows no consistent change in the steroid-resistant Hb12 cells after dexamethasone treatment. The increase at 12-h post-dexamethasone treatment in the WEHI7.2 cells occurs sufficiently early to indicate a signaling event, i.e. before the release of cytochrome *c*, which we begin to detect 16-h post treatment [11].

Increased lysoPtdC can cause apoptosis in some model systems; however, the mechanism is unknown [58,59]. One possibility is that lysoPtdC inhibits the activity of phosphocholine cytidylyltransferase [46] resulting in apoptosis due to a depletion of PtdC [46,60]. The increased PtdC in the dexamethasone-treated WEHI7.2 cells suggests that this is not the mechanism in these cells. Alternatively, increased lysoPtdC can affect membrane fluidity and permeability and act as a detergent [22,58,61]. Although the total percentage of lysoPtdC only reaches ~ 1.5%, well below the 4–5% reportedly needed for a detergent effect [22,61], this does not preclude a local increase above this value. LysoPtdC also interacts readily with proteins affecting their function [22], thus a local lysoPtdC increase may have critical effects on membrane-bound proteins involved in signaling events [58].

Increased lysoPtdC may be an indicator of the buildup of other ^{31}P NMR-invisible, cleavage products that can cause apoptosis. Alterations in enzyme levels, i.e. an increase in phospholipase A_2 (PLA_2), a decrease in lysophospholipase, a decrease in acyltransferase or some combination, may be responsible for the lysoPtdC accumulation. Increased PLA_2 could result in increased free fatty acids (FA), arachidonic acid (AA) or acyl coenzyme A if phospholipid remodeling is also inhibited. Each of these compounds can induce apoptosis [44,62–67] [63–65,67,68]. Several pieces of evidence suggest that AA could be involved in steroid-induced apoptosis. First, the lysoPtdC seen in these spectra is due to a loss of the FA chain at the *sn*-2 position where AA usually occurs [51]. AA is also implicated as the causative agent in non-steroidal anti-inflammatory drug (NSAID)-induced apoptosis in colon carcinoma cells [43,44]. In these cells, NSAIDs work by blocking cyclooxygenase-2 (COX-2) resulting in a buildup of AA and apoptosis. Glucocorticoids are steroidal anti-inflammatory

agents and are known to decrease COX-2 as well [69]. These data also fit with the data from Di Vito et al. [70] where an increase in mobile lipid domains in Jurkat T-lymphocytes during dexamethasone-induced apoptosis was seen. These domains are rich in triglycerides and cholesterol esters, which can act as free FA and AA carriers [71].

Another possibility is that the increase in lysoPtdC seen in the WEHI7.2 cells is the result of an increased ROS attack on membrane phospholipids. Data from our laboratory and others, e.g. Refs. [12,19,72,73], suggest that in lymphocytes increased ROS production after steroid treatment is important in the signaling phase of apoptosis. Increased lysoPtdC in the cells undergoing apoptosis may indicate the presence of lipid peroxidation products, which can bind to and damage DNA and proteins [21] and lead to apoptosis. AA and other polyunsaturated FA, which usually are in the *sn*-2 position, are particularly vulnerable to ROS attack [21]. Spontaneous release of the lipid peroxide product often leaves a short carbon chain in this position. PAF acetylhydrolase, a specialized PLA_2 that degrades platelet-activating factor (PAF), can remove these short chains to generate lysoPtdC [49,74] which would appear in these spectra.

4.2. Dexamethasone and PtdC metabolism

The initial increase in PC along with the maintenance or increase in PtdC suggests that PtdC synthesis is not inhibited and may in fact be stimulated by glucocorticoids. The initial PC increase is followed by a decrease at the later time points. This is in agreement with the dexamethasone-dependent decrease in PME seen by Adebodun and Post [25]. The PC decrease is accompanied by a decrease in Gro-3-P suggesting that the precursors are being consumed. The lack of quantifiable choline and CDP-choline in these samples suggests that dexamethasone does not block choline kinase or phosphocholine DAG transferase.

Hb12 cells show little accumulation of lysoPtdC in the presence of dexamethasone and the greatest increase in GPC, suggesting these cells have an increased ability to degrade phospholipids to PDE. This is supported by the much higher GPE and GPG in these cells even in the absence of dexamethasone treatment. An increased ability to degrade phospholipids may be one way that Bcl-2 provides protection against ROS-induced apoptosis. There are several reports where baseline ROS are higher or an ROS increase is seen after oxidant treatment in bcl-2 transfectants, yet no alteration in lipid peroxidation can be detected [13,14,75,76]. An increased ability to remove damaged lipids could help to explain these data. There is some recent evidence that Bcl-2 family members affect membrane lipid metabolism [77].

4.3. Phospholipids as apoptotic signals

Our data do not support a role for the activation of sphingomyelinase in steroid-induced apoptosis in WEHI7.2 cells. Increased sphingomyelinase activity and production of ceramide has been implicated in steroid-induced lymphocyte apoptosis in the work by Cifone et al. [29]. Dexamethasone treatment causes an increase in PC content which could reflect increased sphingomyelinase activity; however, in WEHI7.2 and CAT38 cells dexamethasone causes an increase in SM rather than the decrease that would be expected if sphingomyelinase was activated [43]. This suggests that sphingomyelinase is not involved, but it is also possible that SM synthesis outstrips demand or activation occurs prior to our measurements.

4.4. Phospholipids and tumorigenesis

Recognition of apoptotic cells in vivo seems to require the externalization of PtdS [35]. In severe-combined-immunodeficient mice, WEHI7.2 cells are least tumorigenic, CAT38 cells are intermediate and Hb12 cells are the most tumorigenetic [10,11]. This difference can be traced to a difference in the rates of spontaneous apoptosis in these tumors. Apoptotic cells from WEHI7.2 cell tumors are readily phagocytized. The increased amount of PtdS seen with dexamethasone treatment in the WEHI7.2 cells and mouse thymocytes [78] could serve to increase recognition of apoptotic cells. The increased AAPtdC and lysoPtdC seen after dexamethasone treatment may also play a role in phagocytosis because one species of the AAPtdC family is PAF; both PAF and lysoPtdC have been shown to stimulate macrophages [79]. In tumors from CAT38 cells, dead cells were not as readily phagocytized as in WEHI7.2 cell tumors. In CAT38 cells PtdS content does not increase with dexamethasone treatment and PtdS externalization is not equivalent to that in WEHI7.2 cells. For example, after 40 h in dexamethasone, the cytochrome *c* release in CAT38 cells is comparable to that in WEHI7.2 cells at 32 h [11], yet the PtdS externalization is much less. We also do not see the AAPtdC increase in these cells. These data suggest that resistance to apoptosis may be accompanied by decreased recognition and phagocytosis of dead cells.

A decreased PC/ΣPDE ratio is correlated with increased resistance to dexamethasone and increased tumorigenicity in these cells. This is primarily due to slightly increased PDE in CAT38 cells and greatly increased PDE values in Hb12 cells. These data suggest that the ability to degrade phospholipids to PDE may be important for both resistance to dexamethasone and tumorigenicity. The appearance of increased lysoPtdC prior to the release of cytochrome *c* into the cytosol suggests that perturbations in PtdC metabolism may be involved in apoptotic signaling. Altered phospholipid metabolism in the resistant variants may either prevent the appearance of these signals or remove them before there is a downstream effect. The variants analyzed in this study provide a good model to further explore these possibilities. Although it is beyond the scope of this manuscript to thoroughly discuss the phospholipid alterations in light of cell cycle alterations and other phenotypic changes in these

cells, these data indicate that overexpression of Bcl-2 or catalase and cell cycle arrest have multiple affects on phospholipid metabolism.

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